REMARKS

In the instant Action, Claims 2-15 are listed as pending, of which Claims 2 and 12-15 are listed as rejected and Claims 3-11 are listed as objected. In reply to the instant Action, Applicant has canceled Claims 2, 13 and 15, and amended Claims 3, 4, 9, 11, 12, and 14, so as to place this application in a condition for allowance.

Applicant gratefully notes that the Examiner has found Applicant's arguments filed 11/21/09 persuasive with the exception of the "ODP" rejection over Claim 1 of USP 7268213.

- Response to nonstatutory obviousness-type double patenting rejection

Claim 2 is rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claim 1 of U.S. Patent No.7,268,213. Without addressing the substantive merits, *vel non*, of this rejection, but solely in order to place the instant application in a condition for allowance, Applicant has canceled Claim 2. In view thereof, Applicant respectfully requests reconsideration and withdrawal of this rejection.

- Response to issues presented under 35 U.S.C. §112, first paragraph

Claims 12-15 are rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Without addressing the substantive merits, *vel non*, of this rejection, but solely in order to place the instant application in a condition for allowance, Applicant has canceled Claim 13 and 15, and amended Claim 14 to be restricted to Type I and Type II diabetes and to depend on Claim 11 reciting exemplified compounds.

With specific regard to Claim 12, which is directed to "a pharmaceutical composition" without any recitation of a disease, it is clear that Claim 12 is outside the Examiner's "first question" of "are the claimed compounds agonists or antagonists of the GLP-1 receptor?". If the Examiner would require further assurance that Claim 12 is

outside the ambit of said question, Applicant is willing to further amend Claim 12 to delete the recitation "pharmaceutical", so as to read as follows:

> 12. (Currently amended) A ~~pharmaceutical~~ composition comprising ~~an effective amount of~~ a compound according to ~~claim 2~~ claim 3 or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier or diluent.

In fact, in the previous Office Action, mailed 05/20/2009, the Examiner raised the "ODP" rejection over U.S. Patent No. 6,903,186, and Claim 2 of U.S. Patent No. 6,903,186 is reproduced below:

> 2. A composition comprising a compound according to claim 1 or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier or diluent.

The point Applicant is trying to make is that a claim directed to "a pharmaceutical composition" comprising a compound already judged to be fully enabled (*See* page 3 of the instant Action wherein the Examiner states that "Claims which are drawn to compounds *per se* are not now being rejected for lack of enablement.") is outside the ambit of the Examiner's "first question" of "are the claimed compounds agonists or antagonists of the GLP-1 receptor?". In view thereof, Applicant respectfully requests reconsideration and withdrawal of this rejection of Claim 12.

With specific regard to Claim 14, Applicant deleted all diseases except Type I diabetes and Type II diabetes, and amended it to depend on Claim 11 reciting exemplified compounds all of which have Aib substitutions at positions 8 and 35. Applicant respectfully submits that the Examiner cannot reject a claim for lacking enablement simply because the inventor does not explain the principle or theory underlying the invention. As stated by the CCPA: "We point out in connection with this rejection that an applicant need not understand the theory or scientific principle underlying his invention. ... All that an applicant need to do is enable a person skilled in the art to duplicate his efforts." *In re Isaacs*, 347 F.2d 887, 146 USPQ 193, 197 (CCPA 1965). It is undisputed that Applicant has disclosed in the instant Application how to synthesize the claimed compound; how to provide the compound in a form suitable for administration to a subject; and even dosage ranges. By accusing Applicant for failing to know if adenylyl cyclase activity is affected one way or another by the claimed

compound, it is clear that the Examiner is applying arbitrary and overly stringent standard of the enablement requirement inconsistent with the substantive requirements of 35 U.S.C. §112, first paragraph.

As noted in M.P.E.P. §2107.03, the federal courts have consistently reversed rejections by the Patent Office asserting a lack of utility for inventions claiming a pharmacological or therapeutic utility where an applicant has provided evidence that reasonably supports such a utility. As the courts have repeatedly held, all that is required is a reasonable correlation between evidence of pharmacological or other biological activity of a compound and the asserted therapeutic use. *Nelson v. Bowler*, 626 F.2d 853, 857, 206 USPQ 881, 884 (CCPA 1980).

For instance, courts have routinely found evidence of structural similarity to a compound known to have a particular therapeutic or pharmacological utility as being supportive of an assertion of therapeutic utility for a new compound. *See* M.P.E.P. §2107.03. In *In re Jolles*, 628 F.2d 1322, 206 USPQ 885 (CCPA 1980), the claimed compounds were found to have utility based on a finding of a close structural relationship to daunorubicin and doxorubicin and shared pharmacological activity with those compounds, both of which were known to be useful in cancer chemotherapy. The evidence of close structural similarity with the known compounds was presented in conjunction with evidence demonstrating substantial activity of the claimed compounds in animals customarily employed for screening anticancer agents. M.P.E.P. §2107.03 mandates that such evidence should be given appropriate weight in determining whether one skilled in the art would find the asserted utility credible.

As discussed in the instant Application, at pages 1-2, GLP-1 is "effective in patients with diabetes (Gutniak, M., N. Engl J Med 226:1316-1322, 1992; Nathan, D.M., et al., Diabetes Care 15:270-276, 1992), normalizing blood glucose levels in type 2 diabetic subjects (Nauck, M.A., et al., Diagbetologia 36:741-744, 1993), and improving glycemic control in type 1 patients (Creutzfeldt, W.O., et al., Diabetes Care 19:580-586, 1996)." The instant Application further provides, at page 2:

> GLP-1 is, however, metabolically unstable, having a plasma half-life ($t_{½}$) of only 1-2 min *in vivo*. Exogenously administered GLP-1 is also rapidly degraded (Deacon, C.F., et al., Diabetes 44:1126-1131, 1995). This metabolic instability

limits the therapeutic potential of native GLP-1. Hence, there is a need for GLP-1 analogues that are more active or are more metabolically stable than native GLP-1.

The claimed compounds of claim 11 differ from the native GLP-1 by the substitution of Aib for Ala at the 8th position and for Gly at the 35th position. Despite these substitutions, all of the compounds of claim 11 are shown to have the ability to competitively bind to the GLP-1 receptor.[1] This retention of the ability to bind to the GLP-1 receptor demonstrates that there is substantial structural similarity between the claimed compound and the native GLP-1. That is, had the substitution of Aib for Ala at the 8th position and for Gly at the 35th position effected any significant structural changes, the resulting compounds would have lost its ability to competitively bind to the GLP-1 receptor. The fact that the claimed compounds retain the full receptor potency is convincing evidence that there is significant structural similarity between the claimed compound and the native GLP-1. Furthermore, a skilled artisan would recognize that these substitutions are effected to improve the plasma half-life of the claimed compound, thereby resulting in enhanced *in vivo* activity of the claimed compound, from the teaching of the following two references:

- Mentlein, R., et al., *Eur. J. Biochem.* 214, 829-835 (1993), wherein it is taught that one of the enzymes that are responsible for the fast degradation of GLP-1 *in vivo* is DPP-IV, which cleaves the amide bond between Ala8 and Glu9 at the N-terminus of hGLP-1; and
- Tammem, H., et al., *J. Chromatogr. A* 852, 285-295 (1999), wherein it is taught that the amide bond between Lys34 and Gly35 of hGLP-1(1-36)NH_2 may also be cleaved *in vivo*.

As such, the skilled artisan would view the substantial structural similarity between the claimed compounds of and the native GLP-1, in conjunction with the aforementioned articles in scientific journals, as competent evidence of the asserted utility of the instant invention.

In further support of the asserted therapeutic efficacy, Applicant has enclosed a copy of an article by Dong, et al., "Glucagon-Like Peptide-1 Analog with Significantly Improved *in vivo* Activity," published in *Peptides: The Wave of the Future, Proceedings*

[1] The data generated via the assay disclosed in the instant Application were previously submitted in reply to the previous Office Action in the form of a Declaration under 37 C.F.R. §1.132 by Dr. John Taylor.

of the Second International and Seventeenth American Peptide Symposium, June 9-14, 2001, San Diego, California, at pages 670-671, wherein it is reported that [$Aib^{8,35}$]hGLP-1(7-36)NH_2,[2] in studies using a mouse model, "enhanced ... insulin response" and was "effective in lowering blood glucose levels." In particular, at page 671 of the article, it is reported that:

> The *in vivo* studies of this new series of hGLP-1 analogs in normal Sprague-Dawley rats demonstrated that the efficacy of the analogs, in terms of the glucose-dependent stimulation of insulin secretion, is highly correlated with their *in vitro* plasma half-life.[3] Among these analogs, compound 4, [$Aib^{8,35}$]hGLP-1(7-36)NH_2, enhanced the insulin response to elevated glucose with a calculated ED_{50} at 16.0 pmol/kg, compared to that of the native hGLP-1(7-36)NH2 at 121 pmol/kg.[4] This 7.6-fold increase in efficacy is likely due to its enhanced enzymatic stability, resulting in an increased circulating half-life. In studies utilizing the *db/db* mouse, intraperitoneal administration of compound 4, [$Aib^{8,35}$]hGLP-1(7-36)NH_2, at 5-50 mmol/kg to 5-week old animals produced a dose-dependent reduction in blood glucose monitored over a 5-h period.[5]
>
> In conclusion, we have designed and synthesized a novel class of GLP-1 analogs that have substantially enhanced plasma half-life, while retaining full receptor potency of the native hormone. The representative analog, compound 4, [$Aib^{8,35}$]hGLP-1(7-36)NH_2, is significant more efficacious than hGLP-1 *in vivo*, and is effective in lowering blood glucose in the *db/db* mouse model of type 2 diabetes.

Clearly, the additional data reported in this article, together with the clear rationale for the correlation between the increased plasma half-life and the efficacy of the claimed compounds which bear structural resemblance to [$Aib^{8,35}$]hGLP-1(7-36)NH_2 to the extent that all of the compounds of claim 11 have Aib substitutions at positions 8 and 35, clearly support the asserted utility of the present invention. As stated in M.P.E.P. §2107.03, "If reasonably correlated to the particular therapeutic or pharmaceutical utility, data

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[2] The discussion involving [$Aib^{8,35}$]hGLP-1(7-36)NH_2 is particular apposite in this context, because Claim 1 of U.S. Patent No. 7,235,628 encompasses Type I and Type II diabetes in conjunction with [$Aib^{8,35}$]hGLP-1(7-36)NH_2, as reproduced here:

> 1. A method for treating a disease selected from the group consisting of Type I diabetes and Type II diabetes in a subject in need thereof, said method comprising administering to said subject an effective amount of a compound according to the formula [$Aib^{8,35}$]hGLP-1(7-36)NH_2(SEQ ID NO:2), or a pharmaceutically acceptable salt thereof.

[3] Culler, M.D., et al. *83rd Annual Meeting of the Endocrine Society*, abstract P1-353, 2001.

[4] Culler, M.D., et al. *83rd Annual Meeting of the Endocrine Society*, abstract P1-360, 2001.

[5] Culler, M.D., et al. *83rd Annual Meeting of the Endocrine Society*, abstract P1-360, 2001.

generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process."

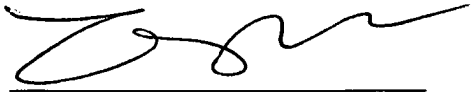
In sum, Applicant respectfully submits that the evidence of close structural similarity, in conjunction with articles in scientific journals and evidence demonstrating substantial activity of the claimed compound in the *db/db* mouse model, should be sufficient to dispel any lingering doubt of the Examiner as to the objective truth of the statements in the disclosure. Accordingly, Applicant respectfully requests reconsideration and withdrawal of the rejection of claim 14 under Section 112, first paragraph.

- Response to issues presented under 35 U.S.C. §112, second paragraph

Claims 12-13 are rejected under 35 U.S.C. §112, second paragraph, as allegedly failing to particularly point out and distinctly claim the subject matter which application regards as the invention. Without addressing the substantive merits, *vel non*, of this rejection, but solely in order to place the instant application in a condition for allowance, Applicant has canceled Claim 13 and amended Claim 12 to delete the recitation "an effective amount of". In view thereof, Applicant respectfully requests reconsideration and withdrawal of this rejection.

In view of the amendments and remarks herein, Applicant respectfully requests that the rejections set forth in the instant Action be reconsidered and withdrawn and that this application be passed to issue. Prompt and favorable action is earnestly solicited.

Respectfully submitted,



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Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7–36)amide, peptide histidine methionine and is responsible for their degradation in human serum

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Peptides of the glucagon/vasoactive-intestinal-peptide (VIP) peptide family share a considerable sequence similarity at their N-terminus. They either start with Tyr-Ala, His-Ala or His-Ser which might be in part potential targets for dipeptidyl-peptidase IV, a highly specialized aminopeptidase removing dipeptides only from peptides with N-terminal penultimate proline or alanine. Growth-hormone-releasing factor(1–29)amide and gastric inhibitory peptide/glucose-dependent insulinotropic peptide (GIP) with terminal Tyr-Ala as well as glucagon-like peptide-1(7–36)amide/insulinotropin [GLP-1(7–36)amide] and peptide histidine methionine (PHM) with terminal His-Ala were hydrolysed to their des-Xaa–Ala derivatives by dipeptidyl-peptidase IV purified from human placenta. VIP with terminal His-Ser was not significantly degraded by the peptidase. The kinetics of the hydrolysis of GIP, GLP-1(7–36)amide and PHM were analyzed in detail. For these peptides K_m values of 4–34 μM and V_{max} values of 0.6–3.8 μmol · min^{-1} · mg protein^{-1} were determined for the purified peptidase which should allow their enzymic degradation also at physiological, nanomolar concentrations. When human serum was incubated with GIP or GLP-1(7–36)amide the same fragments as with the purified dipeptidyl-peptidase IV, namely the des-Xaa–Ala peptides and Tyr-Ala in the case of GIP or His-Ala in the case of GLP-1(7–36)amide, were identified as the main degradation products of these peptide hormones. Incorporation of inhibitors specific for dipeptidyl-peptidase IV, 1 mM Lys-pyrrolidide or 0.1 mM diprotin A (Ile-Pro-Ile), completely abolished the production of these fragments by serum. It is concluded that dipeptidyl-peptidase IV initiates the metabolism of GIP and GLP-1(7–36)amide in human serum. Since an intact N-terminus is obligate for the biological activity of the members of the glucagon/VIP peptide family [e. g. GIP(3–42) is known to be inactive to release insulin in the presence of glucose as does intact GIP], dipeptidyl-peptidase-IV action inactivates these peptide hormones. The relevance of this finding for their inactivation and their determination by immunoassays is discussed.

Dipeptidyl-peptidase IV (DPP IV) is a highly specialized aminopeptidase removing dipeptides from bioactive peptides and synthetic peptide substrates provided that proline or alanine are the penultimate N-terminal residues (Mentlein, 1988, for review). Small peptides or chromogenic substrates with proline in this position are far better hydrolysed than those with alanine (Heins et al., 1988). DPP IV occurs in human serum, as an ectoenzyme on the surface of capillary endothelial cells, at kidney brush-border membranes, on the surface of hepatocytes (here termed also GP110 or OX-61 antigen), on the surface of a subset of T-lymphocytes and thymocytes (here termed CD 26, or thymocyte-activating molecule) and other sites (Loijda, 1979; Nausch and Heymann, 1985; Mentlein et al., 1984; McCaughan et al., 1990). The enzyme has been shown to be responsible for the degradation and inactivation of circulating peptides with penultimate proline, like substance P (Heymann and Mentlein, 1978; Ahmad et al., 1992), but also for growth-hormone-releasing factor (GRF) with penultimate alanine (Frohman et al., 1989; Kubiak, 1989; Boulanger et al., 1992). [Ala^{15}]GRF(1–29)amide with penultimate Ala is even a comparably good substrate as a synthetic Pro^2-containing derivative for purified DPP IV (Bongers et al., 1992). This suggests that the conformation or chain length may greatly influence the cleavage of peptides with penultimate proline/alanine-residues by DPP IV.

We therefore evaluated whether or not other peptide hormones related to GRF might be substrates for DPP IV, and whether this probable proteolytic degradation might be of relevance in the circulation. GRF belongs to the glucagon/

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Abbreviations. DPP IV, dipeptidyl-peptidase IV; GIP, gastric inhibitory polypeptide or glucose-dependent insulinotropic polypeptide; GLP-1(7–36)amide, glucagon-like peptide-1(7–36)amide or insulinotropin or preproglucagon(78–107)amide; GLP-2, glucagon-like peptide-2 or preproglucagon(126–159); GRF, growth-hormone-releasing factor/hormone; PHI, peptide histidine isoleucine; PHM, peptide histidine methionine; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate-cyclase-activating polypeptide.

Enzyme. Dipeptidyl peptidase IV (EC 3.4.14.5).

				5				10			
Tyr	Ala	Asp	Ala	Ile		Phe	Thr	Asn	Ser	Tyr	...-29	*h* GRF(1-29)amide
–	–	Glu	Gly	Thr		–	Ile	Ser	Asp	–	...-42	*h* GIP
His	–	Glu	Gly	Thr		–	Thr	Ser	Asp	Val	...-30	*h* GLP-1(7-36)amide
His	–	–	Gly	Ser		–	Ser	Asp	Glu	Met	...-34	*h* GLP-2
His	–	–	Gly	Val		–	–	Ser	Asp	Phe	...-27	*h* PHM-27
His	–	–	Gly	Val		–	–	Ser	Asp	–	...-27	*r* PHI-27
His	Ser	–	–	Val		–	–	Asp	Asn	–	...-28	*h* VIP
His	Ser	–	Gly	Thr		–	–	Ser	Glu	Leu	...-27	*h* Secretin
His	Ser	Gln	Gly	Thr		–	–	Ser	Asp	–	...-29	*h* Glucagon
His	Ser	–	Gly	–		–	–	Asp	–	–	...-38	*h* PACAP-38

Fig. 1. N-terminal sequences of peptides related to growth hormone-releasing factor (GRF). Penultimate alanine and serine residues are in bold; N-terminal tyrosine and histidine residues are underlined; (–) identity to GRF. *h*, Human sequences; *r*, rat sequence.

secretin/vasoactive-intestinal-peptide(VIP) peptide family (Fig. 1) which share N-terminal sequences of considerable similarity. A number of them begin with Tyr-Ala, namely GRF and gastric inhibitory polypeptide/glucose-dependent insulinotropic peptide (GIP), or with His-Ala, namely glucagon-like peptide-1(7–36)amide/insulinotropin [GLP-1(7–36)amide], glucagon-like peptide-2 (GLP-2), peptide histidine methionine (PHM) and peptide histidine isoleucine (PHI, the rat counterpart of human PHM), whereas others have terminal His-Ser (VIP and others). For biological activity the N-terminal moiety is supposed to be the determinant for transducing the ligand message and the C-terminal moiety for playing the major role in specific binding (Christophe et al., 1989, for review). Thus, proteolytic truncation of the N-terminus of the members of the glucagon/VIP family by DPP IV should inactivate them.

EXPERIMENTAL PROCEDURES

Peptides, inhibitors and enzymes

Synthetic peptide hormones (human sequences) were obtained from Saxon Biochemicals, dipeptides and diprotin A were purchased from Bachem. Purity of peptides was checked by HPLC; their amino acid compositions were analyzed by the manufacturer. Lys-pyrrolidide was a gift from Dr. Mike Schutkowski, Martin-Luther-Universität Halle/Saale, Germany. Dipeptidyl-peptidase IV was purified from human placenta and free of contaminating proteases (Püschel et al., 1982).

Degradation assays with purified enzyme

5 nmol of the peptides (5 µl of a 1 mM solution in water) were incubated at 37°C with 0.1 µg peptidase in 50 mM triethanolamine/HCl, pH 7.8, for 10–60 min in 500 µl (final peptide concentration 10 µM) or less (other peptide concentrations). Enzymic reactions were terminated by addition of 5 µl 10% trifluoroacetic acid, and the mixtures applied onto a Vydac C_{18} widepore (30-nm pores, 5-µM particles) 250 mm × 4.6 mm HPLC column and eluted at a flow rate of 1 ml/min with gradients of acetonitrile in 0.1% trifluoroacetic acid. Either a linear gradient of 0–80% acetonitrile formed within 42 min (GIP degradation), or a stepwise linear gradient of 0–32% acetonitrile formed in 17 min followed by linear gradient of 32–48% acetonitrile formed in 30 min (other peptides) were used for separations. In some HPLC separations, trifluoroacetic acid was replaced by heptafluorobutyric acid. Peptides and their degradation products were monitored by their absorbance at 220 nm (peptide bonds) or 280 nm (aromatic amino acids). They were quantified by integration of their peak areas related to those of standards (synthetic Tyr-Ala or turncated peptides made by complete dipeptidyl-peptidase IV digestion). The concentrations of all peptide solutions were routinely calculated from their absorbance at 280 nm relative to their content of Trp and Tyr (using additively the known absorption coefficients).

Activities were determined from estimations with less than 10% substrate turnover. Catalytic constants were calculated according to the statistical method of Wilkinson (1961).

Degradation of peptides in serum

200 µl serum of healthy males were incubated with 10 µl 1 mM peptide solution in water (final concentration 20 µM) for 60 min at 37°C. Inhibitors were added as 10 mM or 100 mM stock solutions in water. Enzymic reactions were terminated by addition of 20 µl 10% trifluoroacetic acid. Samples were centrifuged (5 min 13000 × *g*), and the supernatant liquids applied to a C_{18} reverse-phase Sep-Pak cartridge (Millipore-Waters) that had been previously activated and washed with 10 ml each of methanol, 80% acetonitrile in 0.1% trifluoroacetic acid and finally 0.1% trifluoroacetic acid. After washing of the serum-loaded cartridges with 20 ml 0.1% trifluoroacetic acid, peptides were eluted with 2 ml 80% acetonitrile in 0.1% trifluoroacetic acid. Acetonitrile eluates were lyophilized, dissolved in 100 µl 0.1% trifluoroacetic acid and analyzed as described above. Non-bound supernatants and washings were combined, lyophilized, reacted with 4-dimethylaminoazobenzene-4-sulphonyl chloride and separated by reverse-phase HPLC as described by Stocchi et al. (1985) for amino acids.

Peptide chemistry and other assays

Fragments separated by HPLC were collected and lyophilized for chemical determinations. Amino acid composition was determined by acid hydrolysis (6 M HCl *in vacuo* at 100°C for 24 h) followed by lyophilisation, reaction with 4-dimethylaminoazobenzene-sulphonyl-chloride and HPLC separation of derivatized amino acids (Stocchi et al., 1985). N-terminal amino acids were determined by manual microsequencing with 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate (Wittmann-Liebold et al., 1986).

Hydrolysis of 0.5 mM Gly-Pro-4-nitranilide at pH 8.6 and at 37°C was monitored as described (Mentlein and Struckhoff, 1989).

RESULTS

Digestion of peptides by purified DPP IV

DPP IV purified from human placenta liberated Tyr-Ala from GRF(1–29)amide and GIP, and His-Ala from GLP-1(7–36)amide and PHM (Table 1, Fig. 2). No further proteolytic cleavage of these peptides was observed indicating the high specificity of the DPP IV for N-terminal Xaa-Ala (and Xaa-Pro) and the absence of contaminating proteases in the enzyme preparation. Liberated Tyr-Ala (Fig. 2) was identified by its retention time and co-chromatography with a synthetic standard. His-Ala was adsorbed to the C_{18} column

Table 1. Cleavage rates for proteolysis of peptides from the GRF/VIP family by DPP IV purified from human placenta. Data are means of three determinations, variations were less than 10%.

Peptide	Concentration	Cleavage rate
	μM	μmol · min^{-1} · mg^{-1}
GRF(1−29)amide	20	4.4
	150	5.0[a]
GRF(1−44)amide	150	4.5[a]
GIP	20	1.4
	100	2.9
GLP-1(7−36)amide	20	0.79
	100	0.35
PHM	20	0.47
	100	0.58
VIP	20	<0.02
	100	<0.02

[a] Data taken from Bongers et al. (1992).

only with heptafluorobutyric acid as ion-pairing reagent (Table 2) which, however, resulted in a relatively high background. Therefore, liberated His-Ala was also identified as its 4-dimethylaminoazobenzene-sulphonyl-derivative (obtained also with a synthetic dipeptide standard). Moreover, the truncated peptides could be separated from the non-degraded ones in reverse-phase HPLC (Fig. 2, Table 2).

Highest initial velocities for DPP-IV degradation at micromolar peptide concentrations were found for GRF(1−29)amide, whereas those for other members of the VIP/glucagon-related peptides with penultimate Ala were lower (Table 1). No significant cleavage was observed with VIP tested as a representative member of this peptide family with N-terminal His-Ser. DPP IV hydrolysed GIP, GLP-1(7−36)amide and PHM with K_m values in the range 4−34 μM (Table 3). These values are of the same order of magnitude as those determined earlier for the cleavage of other bioactive peptides with N-terminal Xaa-Pro or Xaa-Ala by DPP IV. K_m values in the micromolar range have been generally found for other peptide-degrading proteases. Therefore, degradation rates at physiological peptide concentrations in the nanomolar ranges are given by the rate (specificity) constants k_{cat}/K_m. High rate constants indicate high cleavage rates at nanomolar concentrations (below K_m value). k_{cat}/K_m values of about $10^5\,M^{-1}\cdot s^{-1}$ for GIP, GLP-1(7−36)amide and PHM (Table 3) are lower than those determined earlier for good DPP-IV substrates like substance P, but still high enough to ensure a physiological action.

Degradation of GIP and GLP-1(7–36)amide by human serum

When human serum was incubated with 20 μM GIP, two major degradation products were observed (Fig. 3): one eluting at the position of Tyr-Ala, the other at that of des-Tyr−Ala-GIP. Identity of these peaks was ensured by identical retention times with standards (prepared by digestion with pure DPP IV) as well as by amino-acid analysis of the Tyr-Ala peak and determination of the N-terminal amino acid of the GIP (3−42)-peak, both collected after separation. Moreover, addition of the DPP-IV inhibitors 1 mM Lys-pyrrolidide or 0.1 mM diprotin A abolished the generation of both GIP fragments by human serum nearly completely (residual areas <5%). Hydrolysis of 0.5 mM Gly-Pro-4-nitranilide (an established chromogenic substrate of DPP IV) in the same serum sample was reduced to 2% in the presence of 1 mM Lys-pyrrolidide and to 9% after addition of 0.1 mM diprotin A. Lys-pyrrolidide (Lys-tetrahydropyrrole), a substrate analog, and diprotin A (Ile-Pro-Ile), a bad, but high-affinity (K_m = 4 μM) substrate (Rahfeld et al., 1991a), are competitive inhibitors specific (as far tested) for DPP IV. Concluded from their influence and from the fragments generated, GIP

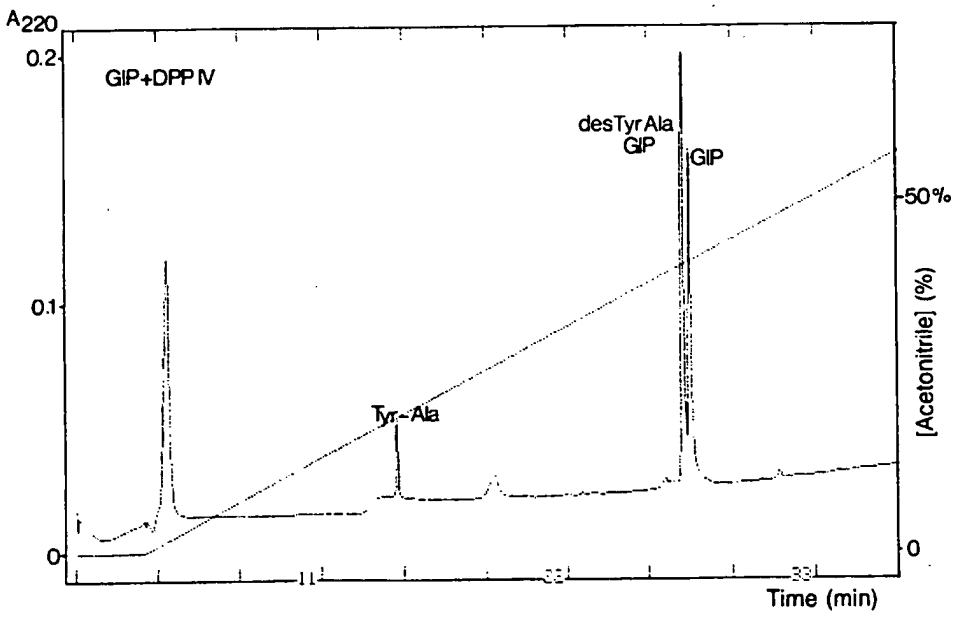

Fig. 2. Reverse-phase HPLC separation of an incubations of GIP with DPP IV purified from human placenta. The positions of liberated Tyr-Ala and of the truncated peptide hormone are indicated. The C_{18} HPLC column was eluted with a gradient of 0−80% acetonitrile in 0.1% trifluoroacetic acid as described in Experimental Procedures. Peptides were monitored in the eluate by their absorbance at 220 nm.

Table 2. Separation of DPP IV cleavage products of gastric inhibitory polypeptide (GIP), glucagon-like peptide-1(7–36)amide [GLP-1(7–36)amide] and peptide histidine methionine (PHM) by reverse-phase HPLC on a C_{18} column. For conditions see Experimental Procedures, retention times varied ±0.3 min. The first 20 min of gradients are identical.

Peptide	Retention time	Gradient
	min	
GIP	27.4	0–3 min 0% + 3–45 min 0–80% acetonitrile in 0.1% trifluoroacetic acid
GIP(3–42)	27.1	
Tyr-Ala	14.3	
His-Ala	3.8	
	18.2	0–3 min 0% + 3–45 min 0–80% acetonitrile in 0.1% heptafluorobutyric acid
GLP-1(7–36)amide	40.7	0–3 min 0% + 3–20 min 0–32% + 20–50 min 32–48% acetonitrile in 0.1% trifluoroacetic acid
GLP-1(9–36)amide	41.7	
PHM	44.1	
PHM(3–27)	44.8	
VIP	35.2	
His-Ala	3.8	

Table 5. Catalytic constants for the degradation of bioactive peptides by human DPP IV. Assays were performed in 50 mM triethanolamine/HCl, pH 7.8, at 37°C. Values of k_{cat} were calculated using a molecular mass of 120 kDa for one identical subunit of the human placental DPP IV dimer (Püschel et al., 1982). GLP-1(7–36)amide shows substrate inhibition above 50 µM, catalytic constants (±SD) were calculated from the linear ranges of Lineweaver-Burk plots.

Peptide	N-terminus	S_0	No. of runs	K_m	V_{max}	k_{cat}	k_{cat}/K_m	Reference
		µM		µM	µmol · min^{-1} · mg^{-1}	s^{-1}	$M^{-1} \cdot s^{-1}$	
GIP	YA-E...	1–100	7	34 ± 3	3.8 ± 0.2	7.6	$0.22 \cdot 10^6$	this study
GLP-1(7–36)-amide	HA-E...	5–100	7	4.5 ± 0.6	0.97 ± 0.05	1.9	$0.43 \cdot 10^6$	this study
PHM	HA-D...	5–100	6	6.5 ± 0.5	0.62 ± 0.03	1.2	$0.19 \cdot 10^6$	this study
[Ala^{15}]GRF(1–29)-amide	YA-D...	2–350	12	4.7 ± 0.3	4.7 ± 0.1	9.5	$2.0 \cdot 10^6$	Bongers et al., 1992
β-Casomorphin	YP-F...	20–500		59	90	180	$3.1 \cdot 10^6$	Nausch et al., 1990
Substance P	RP-K...	25–200		22	10	20	$0.91 \cdot 10^6$	Nausch et al., 1990

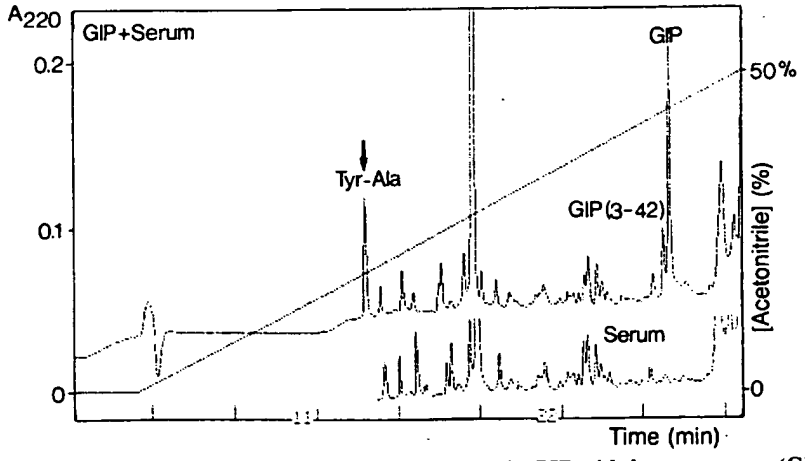

Fig. 3. Reverse-phase HPLC analysis of an incubation assay of 20 µM gastric GIP with human serum (GIP + Serum) compared to a serum blank (Serum, inset). Positions of GIP and its degradation products Tyr-Ala and GIP(3–42) are indicated. Experimental conditions as in Fig. 2.

is metabolized by DPP IV activity of human serum mainly to Tyr-Ala and GIP(3–42).

Incubation of human serum with 20 µM GLP-1(7–36)amide yielded one degradation product at the position of the des-His–Ala-peptide after reverse-phase HPLC (not shown). This fragment was identified by identical retention time with a standard (obtained with pure DPP IV, Table 2) and by determination of the N-terminal amino acid. His-Ala

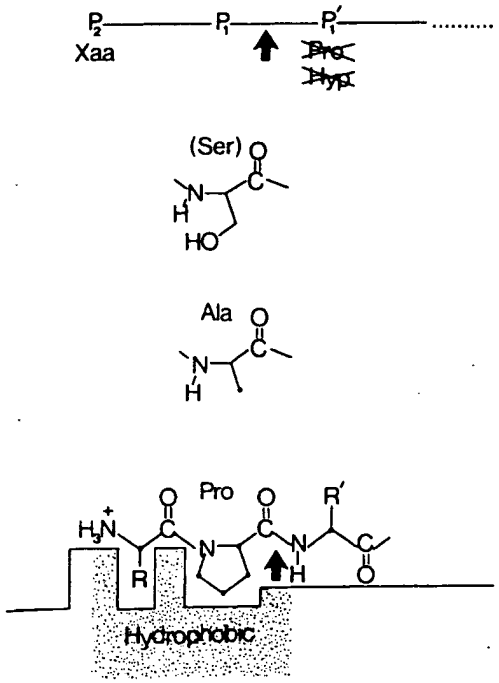

Fig. 4. Schematic representation of the substrate-binding and substrate-cleaving (arrow) sites of DPP IV. Proline and alanine fit in the hydrophobic P_1-substrate-binding pocket, whereas serine appears to be too hydrophilic to yield appreciable binding. In the P_2 position bulky amino acids with an obligate free amino group are preferred. Peptides with Pro or Hyp in the P_1' position are not cleaved by DPP IV. Preferential amino acids for the P_1' position are not known.

as further degradation product could be identified after derivatisation with 4-dimethylaminoazobenzene-sulphonyl-chloride (see Experimental Procedures) by identical retention time and co-chromatography with a derivatized, synthetic His-Ala standard. Again, in the presence of Lys-pyrrolidide (1 mM) and diprotin A (0.1 mM), the generation of the des-His−Ala-fragment was abolished (<5%). Thus, as concluded from specific inhibition and generation of His-Ala and the des-His−Ala-peptide GLP-1(7−36)amide is cleaved by human serum mainly by action of DPP IV.

In sera of healthy males we measured a mean activity of 55 ± 12 μmol · min^{-1} · l^{-1} (n = 6) with the chromogenic substrate 0.5 mM Gly-Pro-4-nitranilide for DPP IV. No significant differences were found for the peptidase activities in preprandial an postprandial sera (n = 3). In a serum with an activity of 50 μmol · min^{-1} · l^{-1} for Gly-Pro-4-nitranilide, we estimated degradation rates of about 0.3 μmol · min^{-1} · l^{-1} for Tyr-Ala liberation from 20 μM GIP and 0.4 μmol·min^{-1} · l^{-1} for His-Ala liberation from 20 μM GLP-1(7−36)amide.

DISCUSSION

Members of the VIP/glucagon peptide family with N-terminal penultimate alanine are good substrates for DPP IV. GRF(1−29)amide or GRF(1−44)amide as analyzed here and by Bongers et al. (1992), GIP, GLP-1(7−36)amide and PHM are cleaved to their des-Tyr−Ala or des-His−Ala derivatives by the highly purified human enzyme. In contrast, VIP with N-terminal His-Ser was not significantly degraded. This fits well with the known, preferential specificity of DPP IV for penultimate proline or alanine residues (Fig. 4). Almost no other naturally occurring amino acid is accepted in this position. Replacement of penultimate Ala in a GRF(1−29)amide derivative by hydrophilic Ser or Gly resulted in dipeptidyl-peptidase-IV substrates of far lower k_{cat} and higher K_m values (Bongers et al., 1992). In contrast, substrates with synthetic hydrophobic derivatives of the proline ring (oxa- or thia derivatives) or short, unbranched hydrophobic alkyl derivatives in the P_1 position are good substrates for DPP IV (Rahfeld et al., 1991b; Schutkowski, 1991). This indicates a hydrophobic substrate (P_1) recognition site for DPP IV where Ser is less well (or not) bound than Ala or Pro (Fig. 4). Moreover, a bulky N-terminal amino acid with free amino group (P_2 position) as with Tyr or His in the peptides investigated here is optimal for high DPP-IV activity. This together with effects of the C-terminal part of the peptides might account for the relatively low K_m and high k_{cat} values of DPP IV for the 29−42 residue hormones GRF, GIP, GLP-1(7−36)amide and PHM as compared to those found earlier for small chromogenic substrates with penultimate Ala (Heins et al., 1988).

GIP released postprandially into the blood from intestinal endocrine K cells inhibits the secretion of gastric acid and stimulates insulin release from pancreatic β-cells in the presence of elevated glucose levels. Schmidt et al. (1986, 1987) have clearly shown that N-terminal Tyr-Ala is absolutely required for the insulin-releasing activity (the main physiological effect) of GIP. Pure des-Tyr−Ala-GIP (3−42) unlike intact GIP did not increase insulin secretion in the presence of 16.7 mM glucose from rat pancreatic islets at physiological or higher concentrations even up to 250 nM. Therefore, truncation of GIP by DPP IV results in its inactivation with respect to its major physiological, the insulinotropic, action.

Cleavage products and influence of specific inhibitors clearly show that dipeptidyl peptidase IV is the main degradation and, considering the above findings, inactivation enzyme for GIP in human serum. The enzyme should be still more active on this peptide hormone at other sites, e. g. endothelial cells of blood vessels, hepatocytes, kidney brush-border membranes (podocytes of the glomerular basement membrane and proximal tubule cells), lymphocytes, chief cells of gastric glands, or epithelial cells of the intestine, where it is found in high concentrations as an ectoenzyme of the plasma membranes (Loijda, 1979; Hartel et al., 1988; Gossrau, 1979; McCaughan et al., 1990; Mentlein et al., 1984). Active hydrolysis by DPP IV might therefore explain why GIP(3−42) has been isolated as a second component (relative yield about 20−30%) beside intact GIP from porcine intestine and has been found as a contaminant of natural GIP preparations (Jörnvall et al., 1981; Schmidt et al., 1987).

GLP-1(7−36)amide is a product of the tissue-specific post-translational processing of the glucagon precursor. It is released postprandially from intestinal endocrine L cells and stimulates insulin secretion. Gallwitz et al. (1990) have shown that the C-terminal fragment of the peptide is important for receptor binding of the hormone, but is not sufficient to transduce a biological action as does the intact peptide (raise in cyclic AMP levels in rat insulinoma RINm5F cells). It appears that as in the case of glucagon (Unson et al., 1989), of GIP (Schmidt et al., 1986, 1987) and of other members of the VIP/glucagon peptide family (Christophe et al., 1989; Robberecht et al., 1992) also for GLP-1(7−36)amide an intact N-terminus is needed for signal transduction and biological action. Provided this, action of DPP IV inactivates GLP-1(7−36)amide.

Based on the identification of cleavage products and influence of specific inhibitors, DPP IV is the main degradation enzyme for GLP-1(7−36)amide in human serum. Buckley and Lundquist (1992) have reported recently in an abstract the formation of GLP-1(9−37) by human plasma, but did not identify the peptidase responsible for its generation. As outlined for GIP above, plasma-membrane-bound DPP IV of endothelial and other cells might be still more important for the inactivation of GLP-1(7−36)amide than the plasma activity.

PHM (rat counterpart PHI) and VIP are processing products of a common precursor and are co-released from central and peripheral neurons. As far as is known, PHM/PHI have biological effects similar or identical to VIP. Since it is known that the biological actions of VIP critically depend on an intact N-terminus (Christophe et al., 1989; Robberecht et al., 1990), in analogy also PHM/PHI might be inactivated by cleavage of the N-terminal dipeptide by DPP IV. Since serum concentrations of PHM like VIP are low and in contrast to GIP and GLP-1(7−36)amide do not rise postprandially (Boden and Shelmet, 1986), inactivation in serum is probably of minor importance and was not investigated. It can, however, be suspected that DPP IV cleaves the paracrine acting peptides PHM/PHI in other tissues where it is present on the surface of various epithelial and endothelial cells.

In conclusion, members of the glucagon/VIP peptide family with N-terminal Tyr-Ala or His-Ala, namely GRF, GIP, GLP-1(7−36)amide and PHM, are inactivated by action of DPP IV in human serum. The truncated peptides could also be antagonists, because the binding specificity is directed by the C-terminal parts of these peptide hormones (Christophe et al., 1989; Gallwitz et al., 1990). Since the cleavage by this peptidase removes only 2 of 29−42 total residues of the hormones, antisera against these peptides not directed specially to the N-terminus should cross-react also with the truncated peptides. Therefore, immunoassays for these hormones can be hampered by the measurement of biologically inactive, des-Xaa−Ala forms beside the active peptide, due a potential cross-reactivity of the antisera. Unless specific N-terminally directed antisera are available, serum samples should be stored for immunoassays at least in the presence of DPP-IV inhibitors (specific ones mentioned here or serine protease inhibitors like phenylmethanesulphonyl fluoride).

DPP IV in human serum and at the surface of endothelial cells is known to be involved in the inactivation of other circulating bioactive peptides: removal of the N-terminal tetrapeptide Arg-Pro-Lys-Pro of substance P (Heymann and Mentlein, 1978) inactivates only some biological actions of this neuropeptide (e.g. histamine release from mast cells), but renders the peptide possible for the complete degradation by aminopeptidase M (Ahmad et al., 1992). Several other bioactive peptide with N-terminal Xaa-Pro including gastrin-releasing peptide, corticotrophin-like intermediate lobe peptide and β-casomorphin are excellent substrates for the purified peptidase (Nausch et al., 1990).

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JOURNAL OF CHROMATOGRAPHY A

Proteolytic cleavage of glucagon-like peptide-1 by pancreatic β cells and by fetal calf serum analyzed by mass spectrometry

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Abstract

Fetal calf serum and a β-cell line exhibit a proteolytic activity essential for the biological function of glucagon-like peptide-1 (GLP-1). This process of cleavage was investigated using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). To generate processing products, GLP-1 was subjected to rat insulinoma m5F (RINm5F) cell cultures or to fetal calf serum (FCS). For detection of processing products, a standardized extraction method including ion-exchange batch extraction, ultrafiltration, gel filtration, and reversed-phase chromatography was used. The RP fractions were analyzed by MALDI-TOF-MS. Processed proteolytic products were detected by comparing the resulting mass spectra of cell media or FCS after 2 h incubation with GLP-1 (7–36) amide with these of 2 h controls. To perform the comparison of the resulting mass spectra, software (MASSSPECANALYST) based on Microcal Software, Origins C-like language LABTALK was developed. GLP-1 fragments were purified by RP-HPLC, and characterized by sequence analysis. As insulin is the major secretory product of β cells depending on GLP-1 stimulation, the insulin and insulin fragments of the cell culture supernatants were also analyzed by this method.

Keywords: Peptides; Insulin; Proteins; Glucagon-like peptide-1

1. Introduction

Glucagon-like peptide-1 (GLP-1) was first described by Lund et al. [1]. They sequenced the preproglucagon cDNA of anglerfish and identified two glucagon-like coding sequences arranged in tandem. The presence of Lys–Arg sequence flanking and glucagon-related sequences suggested that two peptides are formed in vivo by post-translational cleavage of this common precursor. Proglucagon is produced in both pancreatic and intestinal endocrine cells. Post-translational processing of the precursor yields different peptides in these organs [2,3]. In the intestine, the major products are glicentin (PG-1-69), oxyntomodulin (PG-33-69), GLP-1 and GLP-2 [4–6].

GLP-1 is involved in the regulation of many body functions. For example, the ability of an oral glucose load to increase insulin secretion at a higher level than an intravenous glucose load is due to the intestinal release of GLP-1 and gastric-inhibitory polypeptide (GIP) by intraluminal glucose stimulation [7–9]. GLP-1 is mostly secreted from the distal ileum in response to mixed meals [10]. GLP-1 stimulates insulin secretion [9].

Clinical studies of diabetic patients show that GLP-1 improves diabetes control [11]. A reduced insulin requirement was observed during GLP-1 treatment.

In the present study, we investigated the

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proteolytic processing of GLP-1 in cell media of β cells and by fetal calf serum using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The processing products may be of importance due to the possible generation of intrinsic biological activities that differ from those of GLP-1 (7–36) amide, e.g. they may be antagonists of GLP-1.

2. Methods

2.1. Cell culture

RINm5F cells were grown in plastic culture bottles with Roswell Park Memorial Institute (RPMI) 1640 medium, 10% fetal calf serum (FCS), 5 m*M* penicillin, 5 m*M* streptomycin, 37°C, air–CO_2 (95:5). Cell concentrations were determined by counting the cells in a Neubauer chamber.

2.2. Incubation of RINmF5 cells with GLP-1

Cells were seeded at a concentration of $0.5 \cdot 10^6$/ml in a 100-ml plastic culture bottle and grown for 48 h. The cells were washed three times with phosphate buffered saline (PBS). Incubation was performed with FCS-free RPMI 1640 medium. GLP-1 stimulation was performed with GLP-1 (7–36) amide at a concentration of 0.6 μ*M* for 2 h. Proteolytic activity of FCS was determined by incubation of 10% FCS-containing RPMI 1640 medium with GLP-1 (7–36) amide at a concentration of 0.6 μ*M* for 2 h in the absence of RINmF5 cells. The medium was collected and centrifuged at 200 *g*. The pH was adjusted to 2.7 using hydrochloric acid and diluted with water to a conductivity of 5 mS/cm.

2.3. Preparation of peptides from RINm5F cell supernatant

Centrifuged RINm5F cell culture medium was loaded on a cation-exchange column (50×4 mm, Fractogel TSK SP 650 M, Merck, Darmstadt). Batch elution of the peptides was achieved with 1 *M* ammonium acetate, pH 9.0. Purification and separation of the peptides is performed using reversed-phase C_{18} chromatography.

In experiments analyzing the proteolytic effect of FCS, the samples were ultrafiltered using an M_r 30 000 cut-off membrane (MiniPlate Amicon, Germany). The pressure gradient of 2 mbar was achieved by using a tube pump. The ultrafiltrate was loaded on a reversed-phase column for batch elution (Source 15 RPC, 125×4 mm, Pharmacia, Freiburg, Germany). The eluate was lyophilized. An equivalent of the lyophilisate was dissolved in 50 μl PBS, loaded on a gel filtration column (Superdex G75; 300×3 mm, Pharmacia) and eluted at a flow-rate of 40 μl/min. Collection of the eluate was started after 1.6 ml to exclude the high-molecular-mass proteins. The low-molecular-mass fraction was loaded on a reversed-phase column for separation (Zorbax MicroTech C_{18}, 150×1 mm, Sunnyvale, CA, USA). The generated fractions were subsequently subjected to MALDI-MS.

2.4. MALDI-MS

A 1-μl volume of each RP fraction and 1 μl of α-cyanohydroxycinnamic acid were applied to a stainless steel multiple sample tray as admixture using the dried drop technique. Measurements were performed in linear mode with a LaserTec RBT MALDI-MS system (Perseptive/Vestec, Houston, USA). The instrument is equipped with a 1.2-m flight tube and a 337-nm nitrogen laser. Positive ions are accelerated at 30 kV and 64 laser shots are automatically accumulated per sample position. The time-of-flight data are externally calibrated for each sample plate and sample preparation. For calibration a mixture of bradykinin (1061.24), human secretin (3040.46), and bovine insulin (5734.59) is used. Data acquisition and first analysis, implying an automatic Savitzky Golay smoothing, baseline correction, and peak labeling, is performed using GRAMS software supplied by the manufacturer.

2.5. Analysis of mass spectrometric data

For further analysis of the MALDI-MS data, new software MASSSPECANALYST based on Microcal Software Origins C-like language LABTALK has been

developed. To perform the comparison of the data, the spreadsheet files of the mass spectra produced by GRAMS software generated from the RP-HPLC fractions are used to prepare a single mass data table. Each mass data table is a list of the detected molecular masses of all fractions in a single preparation. With each molecular mass the according intensity, fraction number and MALDI-MS filename is listed. This database is the basis for further analysis. The data are reduced from ≈1 MB per MALDI mass trace to 70 KB per database.

The software compares two tables in consideration of the error of measurement, elution position of the molecular masses, and intensity of the molecular masses. These parameters are used to determine whether two mass values represent identical molecules. The error of measurement of the LaserTec RBT MALDI-MS is 2‰. The software generates 'mass identity spectra', which represent the molecular masses found in each of two peptide preparations and 'mass difference spectra', which represent the molecular masses found in only one of two peptide preparations.

2.6. Sequence analysis

Sequence analysis of the isolated peptides is performed by stepwise Edman degradation using a gas-phase automated sequencer (Model 470 A, Applied Biosystems, Weiterstadt, Germany). The resulting phenylthiohydantoin (PTH)-amino acids are identified by HPLC.

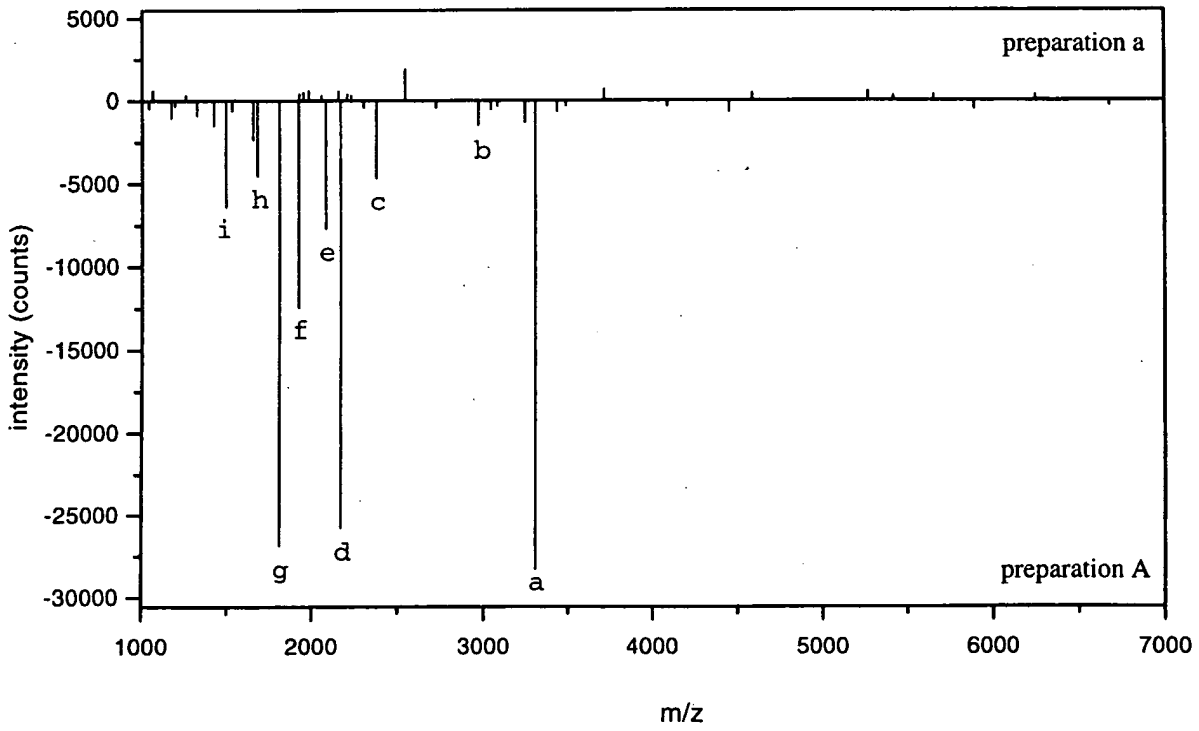

Fig. 1. Mass difference spectrum shows a comparison of the peptide content from unstimulated RINm5F cell supernatant (preparation a) with a preparation of supernatant from GLP-1 (7–36) amide-stimulated RIN m5F cells (preparation A). The masses found in one of the two preparations are shown. The mass data of both supernatants were compared by MASSSPECANALYST software. The letters a to i mark the mass spectrometric peaks representing GLP-1 proteolytic products. The crosses (+) in the legend refer to proteolytic products isolated and sequenced by Edman degradation. [a=3303: GLP-1 (7–36) amide (+); b=2968: GLP-1 (10–36); c=2378: GLP-1 (7–28); d=2170: GLP-1 (18–36) (+); e=2080: GLP-1 (19–36) (+); h=1678: GLP-1 (22–36) (+); g=1810: GLP-1 (21–36) (+); f=1923: GLP-1 (20–36); i=1493: GLP-1 (24–36)].

3. Results

3.1. Identification of rat-insulin-1, GLP-1 and their proteolytic fragments

Serum-free supernatant from unstimulated RINm5F cells as well as GLP-1 stimulated RINm5F cells were produced. To induce r-insulin secretion, 0.6 μ*M* GLP-1 was subjected to the RINm5F culture. Supernatants were subjected to cation-exchange chromatography and batch-eluted. The eluate was fractionated using RP-HPLC chromatography. Fractions were lyophilized and diluted in 100 μl sample buffer. A 1-μl volume of the fractions was subjected to MALDI-MS. The spreadsheet files were processed using the computer software MASSSPECANALYST to prepare a single mass data table for each run of chromatography. MASSSPECANALYST was used to compare the single mass data tables from unstimulated and stimulated RINm5F supernatants. Mass identity and mass difference spectra are shown in Figs. 1 and 2. In the mass identity spectrum, the mass of r-insulin (M_r 5816) was identified. Purification and amino acid sequencing of this mass confirmed r-insulin I. The insulin peak from GLP-1-stimulated RINm5F cells has an intensity 3 times higher than the unstimulated sample. The molecular mass of 2850 was identified as a proteolytic product of r-insulin I (25–49).

Applying the described technique, the culture medium of the GLP-1-stimulated RINm5F cells reveals the different cleavage products of GLP-1 (7–36) amide. According to MS data (Fig. 1), these are GLP-1 (7–36), GLP-1 (10–36), GLP-1 (7–28), GLP-1 (18–36), GLP-1 (19–36), GLP-1 (20–36), GLP-1 (21–36), GLP-1 (22–36), and GLP-1 (24–36). Furthermore, GLP-1 (7–36), GLP-1 (18–36), GLP-1 (19–36), GLP-1 (21–36), and GLP-1 (22–36) are identified by purification and amino acid sequencing (Fig. 1). The mass difference spectrum of Fig. 1 depicts the proteolytic products of GLP-1 (7–36) amide in the form of the most prominent peaks.

Incubation of GLP-1 in FCS (Fig. 6) reveals the

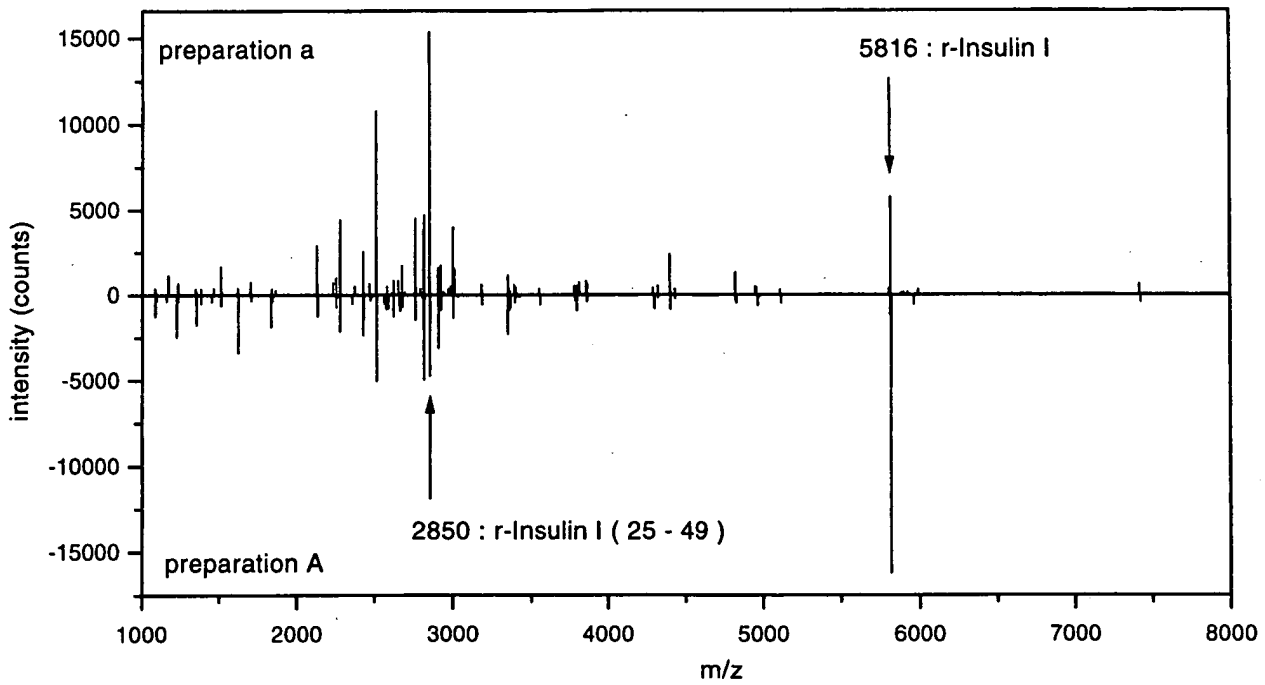

Fig. 2. Mass identity spectrum shows a comparison of the peptide content from unstimulated RINm5F cell supernatant (preparation a) with a preparation of supernatant from GLP-1 (7–36) amide-stimulated RIN m5F cells (preparation A). Comparing the mass data tables of preparation a and A, taking into consideration the error of measurement and the elution time of the peptides, the software MASSSPECANALYST produces this mass identity spectrum, demonstrating the detected masses found in both preparation.

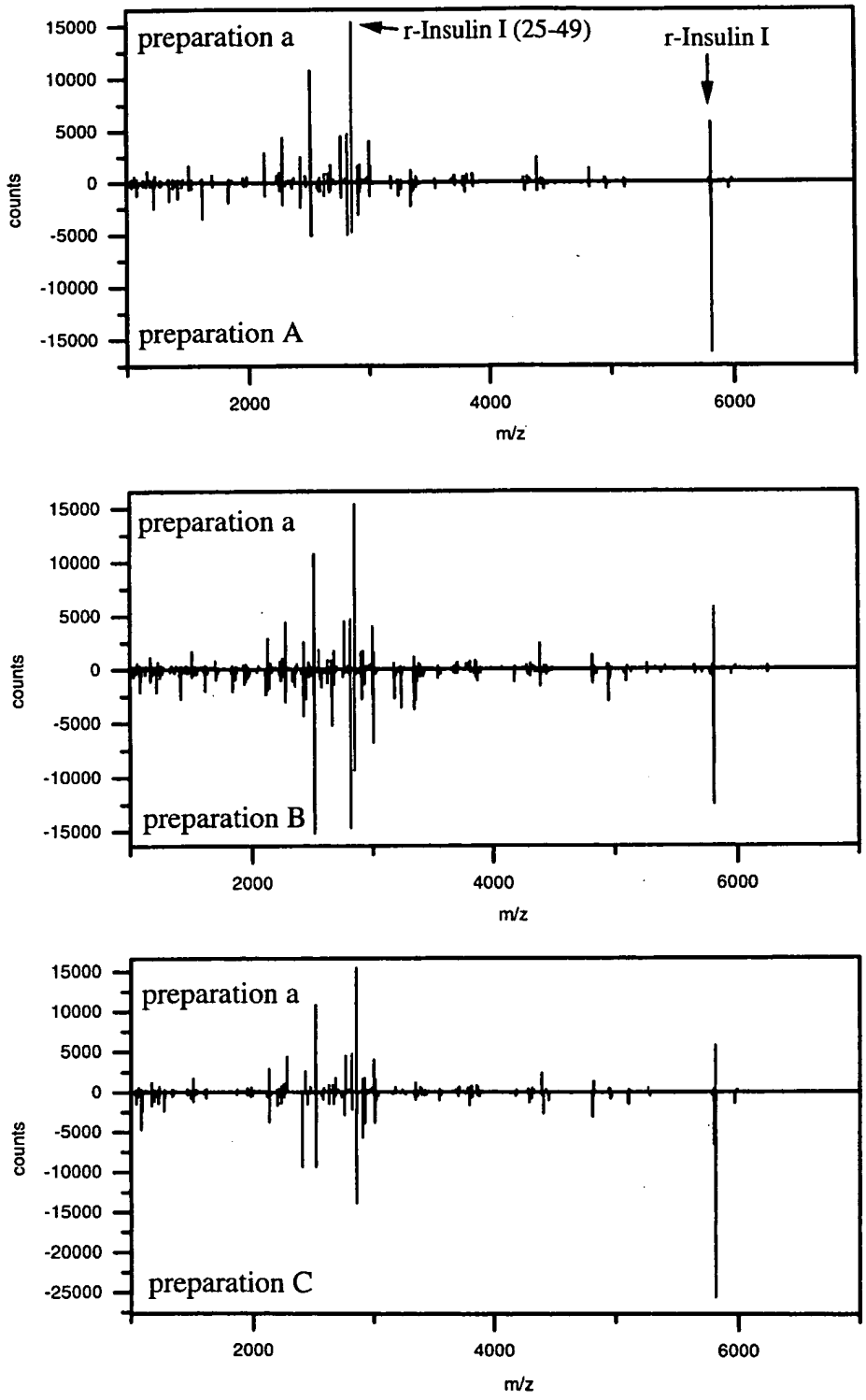

Fig. 3. Three mass identity spectra show a comparison of the peptide content from unstimulated RINm5F cell supernatant (preparation a) with three different preparations of supernatant from GLP-1 (7–36) amide-stimulated RINm5F cells (preparation A, B, C).

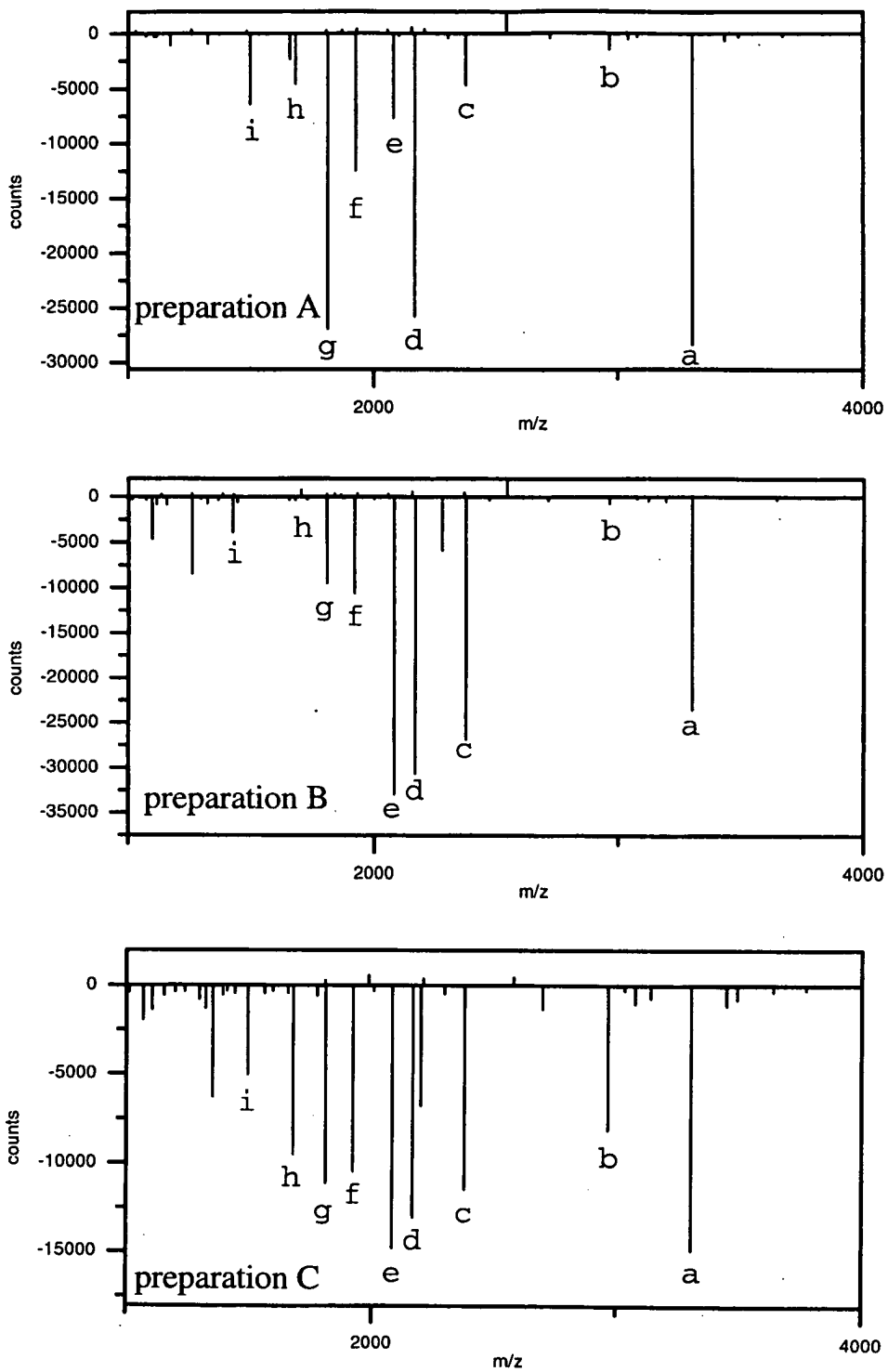

Fig. 4. Three mass difference spectra show a comparison of the peptide content from unstimulated RINm5F cell supernatant (preparation a) with three different preparations of supernatant from GLP-1 (7–36) amide-stimulated RINm5F cells (preparations A, B, C). (Letters refer to the legend in Fig. 2).

mass difference spectrum demonstrating molecular masses, which may represent GLP-1 (22–36), GLP-1 (21–36), GLP-1 (19–36), GLP-1 (9–34), GLP-1 (9–36), GLP-1 (8–36), and GLP-1 (7–36). These peaks presenting the cleavage fragments of GLP-1 are predominant in the mass difference spectrum.

3.2. Reproducibility of the method

For assessment of the reproducibility of the method, three preparations (preparation A, B, C) of RINm5F cell supernatant incubated with GLP-1 (7–36) amide are compared with an unstimulated sample (preparation a) by producing mass identity and mass difference spectra (Figs. 3 and 4). The mass of r-insulin I and the proteolytic fragment (25–49) are detected in each preparation. In all preparations, the insulin peak from GLP-1-stimulated RINm5F cells is prominently higher when compared to the unstimulated sample. The identified proteolytic fragments of GLP-1 (7–36) amide are present in all mass difference spectra.

For further examination of the reproducibility of the method, 23 molecular masses with high intensity found in a preparation of stimulated RINm5F cell supernatant have been selected and their occurrence has been determined in five other preparations. Fig. 5 reveals that 16 out of 23 selected molecular masses are found in all preparations, 2 molecular masses were found in 5, 3 molecular masses are found in 4, and 1 molecular mass is found in only 3 preparations. Missing molecular masses are in a molecular mass range >3500. With the exception of the molecular mass of 5818-representing r-insulin-1- the molecules >4000 are detected only with intensities <2000.

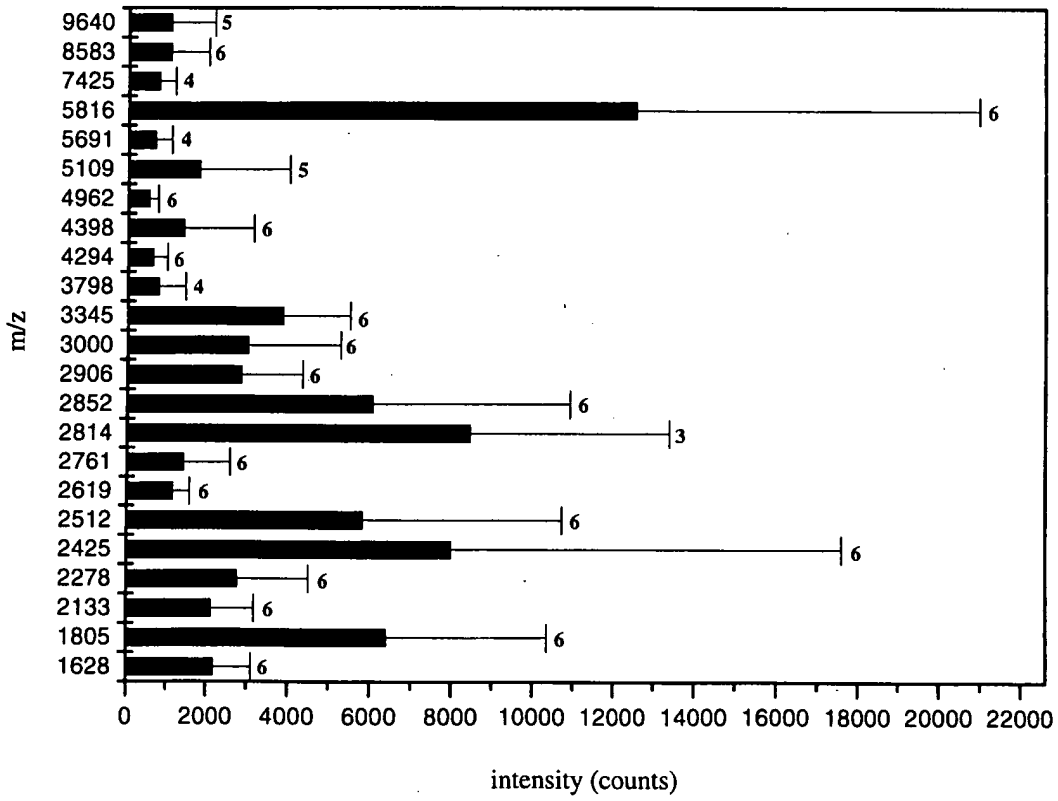

Fig. 5. Mass intensities (mean value and standard error) of masses identified in six preparations. The mass values represent detected MALDI-MS peaks, which appear in different preparations of GLP-1 (7–36) amide incubated supernatant of RIN m5F cells. The columns show the mean intensities of detected masses. The numbers next to the error bars indicate the number of appearances of each mass in six preparations.

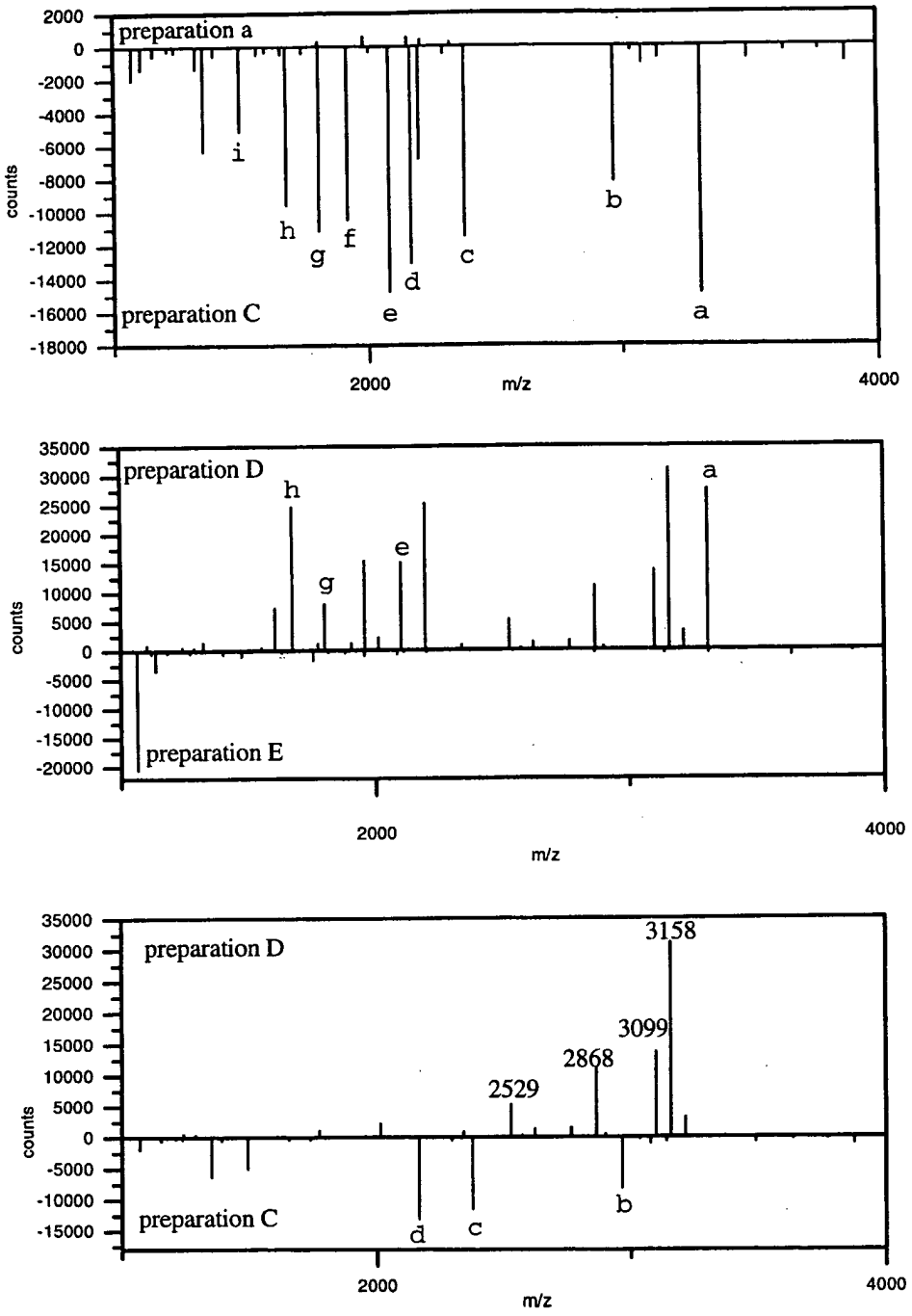

Fig. 6. Identification of cleavage products of GLP-1 (7–36) amide generated in fetal calf serum. The upper mass difference spectrum shows a comparison of the peptide content from unstimulated RIN m5F cell supernatant (preparation a) with a preparation of GLP-1 (7–36) amide stimulated RIN m5F cell supernatant. The second mass difference spectrum shows a comparison of fetal calf serum containing GLP-1 (7–36) amide (preparation D) and 10% fetal calf serum containing media (preparation E). Marked mass spectrometric peaks a, e, g, and h may represent GLP-1 (7–36) amide, GLP-1 (19–36), GLP-1 (21–36), and GLP-1 (22–36) respectively. The last mass difference spectrum shows a comparison of the data gained from both upper spectra. The numbers indicate the detected masses. By using MACBIOSPEC, the only possible cleavage product of GLP-1 for 3158 is GLP-1 (8–36), for 3099 GLP-1 (9–36) and for 2868 GLP-1 (9–34). Marked mass spectrometric peaks b, c, and d refer to GLP-1 (10–36) GLP-1 (7–28), and GLP-1 (18–36), respectively.

3.3. Quantification of GLP-1 (7–34) amide by MALDI signal

To determine the dependence of peptide concentration in the sample and signal intensity in MALDI-MS, different concentrations of GLP-1 (7–34) amide are measured using CHC matrix. 60 fmol/μl of GLP-1 (7–34) amide are necessary to give a reliable determination of the peptide in MALDI-MS (Fig. 8).

4. Discussion

This study was initiated to investigate the proteolytic cleavage of GLP-1 by RINm5F cells and in FCS using MALDI-MS. To carry out this study a new tool, the computer program MassSpecAnalyst was developed to compare the MALDI mass data gained from the chromatographic fractions of RINm5F cell culture supernatant.

GLP-1 fragments	Detected M_r	Theoretical average M_r	Sequenced by Edman degradation
GLP-1 (7-36)	3305.5	3298.6	+++
GLP-1 (7-28)	2377.9	2374.5	
GLP-1 (8-36)	3158.3	3161.5	
GLP-1 (9-36)	3099.4	3090.4	
GLP-1 (9-34)	2868.1	2877.2	
GLP-1 (10-36)	2968.4	2961.3	
GLP-1 (18-36)	2168.6	2166.5	+++
GLP-1 (19-36)	2081.4	2079.4	+++
GLP-1 (20-36)	1923.8	1916.2	
GLP-1 (21-36)	1810.1	1803.1	+++
GLP-1 (22-36)	1678.6	1673.1	+++
GLP-1 (24-36)	1492.9	1488.8	

Fig. 7. (A) Amino acid sequence of GLP-1 (7–36) amide. Arrows (↑) indicate the identified processing sites of GLP-1. (B) Detected molecular masses and theoretical average molecular masses of the identified GLP-1 fragments. Identified GLP-1 fragments using amino acid sequencing, are indicated in the right column.

Using this method, eight different GLP-1 proteolytic fragments were identified in RINm5F cell culture medium. Four of these were isolated and amino acid sequencing confirmed the identity of molecular masses and GLP-1 fragments. GLP-1 fragments induced a high MALDI-MS intensity. Four of the molecular masses were not sequenced, but the high intensity of the peaks as well as the molecular mass itself suggest that the peaks represent GLP-1 (10–36) and GLP-1 (7–28) GLP-1 (20–36), and GLP-1 (24–36).

Interestingly, in our study primarily N-terminally truncated fragments were found with proteolytic cleavage between amino acids 9 and 10, 17 and 18, 18 and 19, 19 and 20, 20 and 21, 21 and 22, and 23 and 24, whereas Hupe-Sodmann et al. [13] found only the C-terminal fragment GLP-1 (29–36). One explanation for this finding is the use of plasma membranes of RINm5F cells in the study of Hupe-Sodmann et al. [13] whereas in our study we used intact RINm5F cells.

Comparison of GLP-1 fragmentation in plasma and in RINm5F cell culture suggest that three GLP-1 fragments be exclusively produced in plasma (Fig. 6). These N-terminally truncated forms are suggested to be GLP-1 (8–36), GLP-1 (9–36), and GLP-1 (9–34). The other identified cleavage fragments appear in both preparations. Interestingly, GLP-1 (9–36) is suggested to be a cleavage product of the dipeptidylpeptidase IV which is known as a plasmatic enzyme and which inactivates the GLP-1 (7–36) amide [12]. In addition, it is suggested that it binds to the GLP-1 receptors and thus may act as a receptor antagonist [12].

Assessment of the reproducibility of the method used shows that r-insulin, an r-insulin fragment,

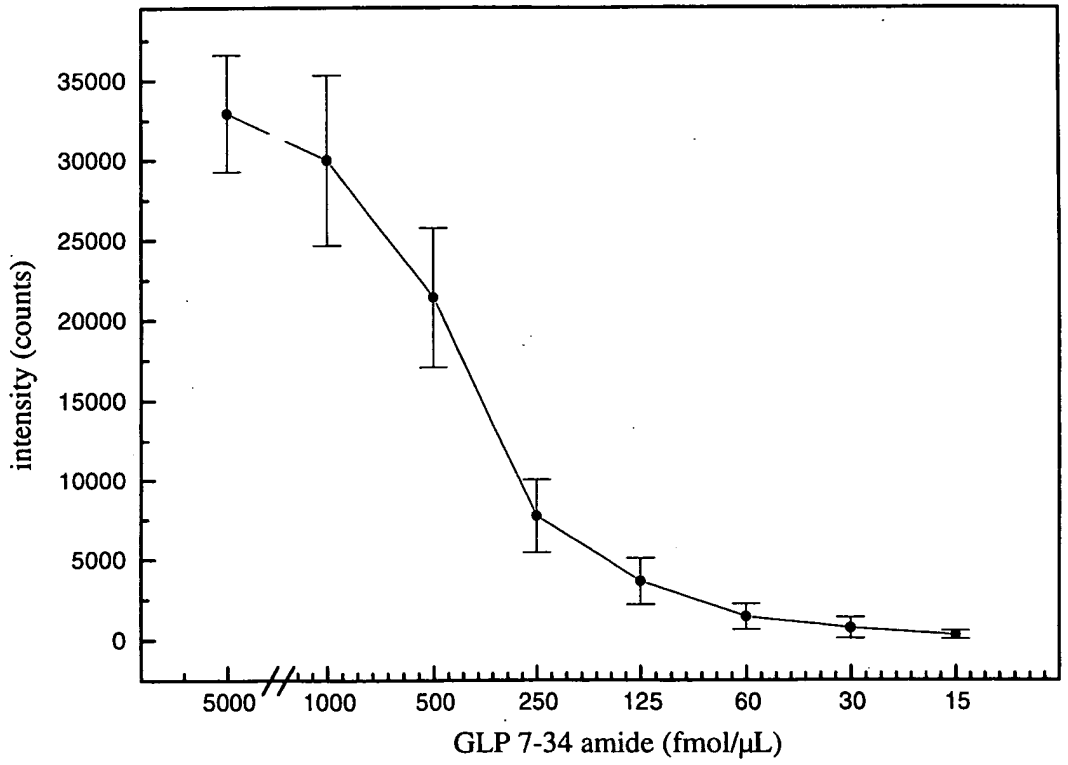

Fig. 8. Dependence of MALDI-MS signal intensity and GLP-1 (7–34) amide concentration. 1 μl GLP-1 (7–34) amide admixtured in 50% (v/v) acetonitrile, 0.1% trifluoroacetic acid were subjected to the multiple sample tray. Eight different concentrations of GLP-1 were analyzed by MALDI-TOF-MS using α-cyano-4-hydroxycinnamic acid as matrix. GLP-1 (7–34) amide concentrations ranged from 15 fmol/μl to 5 pmol/μl. Each data point represents the mean value and the standard error of eight trials.

GLP-1 (7–36) amide, as well as eight GLP-1 fragments are detected in three preparations (Figs. 3 and 4). Furthermore, of twenty-three molecular masses, sixteen are found in each of six preparations of RINm5F cell supernatants (Fig. 5). Seven masses are found only in three, four and five preparations. An explanation for the missing molecular masses as well as the high standard errors of the peak intensities is the sample preparation, which starts with incubation of the cell culture, followed by cation-exchange chromatography, RP chromatography, lyophilization of the samples and manual pipetting of the MALDI plates (Fig. 7). Measurement of GLP-1 (7–34) amide standards revealed a concentration dependence of the MALDI signal intensity suggesting that a quantification of the amount of peptide in a sample is possible (Fig. 8).

Our study shows a new strategy for the complex analysis of reduced MALDI-TOF-MS mass spectra. The application of this method allows the examination of the molecular fragments of regulatory peptides appearing during metabolism and processing in vitro by HPLC and MALDI-MS.

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Peptides

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Glucagon-Like Peptide-1 Analogs with Significantly Improved *in vivo* Activity

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Introduction

Glucagon-like peptide-1 (GLP-1), a potent and strictly glucose-dependent insulinotropic agent, has received increasing attention as a possible new treatment for type 2 diabetes. Although its effectiveness in type 2 diabetes patients has been demonstrated in clinical evaluations, the potential use of the native GLP-1 as a therapeutic agent is greatly hampered by its short plasma half-life. Physiologically, GLP-1 is rapidly degraded by endoproteases. Here we report that a series of novel human GLP-1 (hGLP-1) analogs have been designed and synthesized, which have greatly improved plasma half-life and significantly enhanced *in vivo* activity.

Results and Discussion

One of the enzymes that are responsible for the fast degradation of GLP-1 *in vivo* is DPP-IV, which cleaves the amide bond between Ala8 and Glu9 at the N-terminus of hGLP-1 [1]. To prevent this enzymatic cleavage, we replaced Ala8 with some unnatural amino acids, including *N*-methyl-D-alanine (N-Me-D-Ala), 1-aminocyclopentane-1-carboxylic acid (A5c), and aminoisobutyric acid (Aib). These sterically hindered amino acids make the peptide bond between positions 8 and 9 less accessible to the enzyme, yielding analogs with greater DPP-IV resistance (compounds **1**, **2**, and **3**, Table 1).

Knowing that the amide bond between Lys34 and Gly35 of hGLP-1(1-36)NH_2 may also be cleaved *in vivo* [2], we further substituted the C-terminal Gly35 residue with Aib or β-alanine (β-Ala) with the goal of protecting the peptide bond. The resulting analogs bearing modifications at both positions 8 and 35 (compounds **4**–**8**, Table 1) have much longer plasma half-life than mono-substituted compounds **1**, **2** and **3**. These

Table 1. hGLP-1 receptor binding affinity and rat plasma half-life.

Peptide	hGLP-1[a] Ki (nM)	Rat plasma T1/2 (h)	Sequence
hGLP-1(7-36)NH_2	1.09	0.84	
1	1.13	4.35	[N-Me-D-Ala^8]hGLP-1(7-36)NH_2
2	7.23	4.86	[A5c^8]hGLP-1(7-36)NH_2
3	0.64	4.52	[Aib^8]hGLP-1(7-36)NH_2
4	0.95	9.76	[Aib^{8,35}]hGLP-1(7-36)NH_2
5	1.26	8.34	[Aib^8,β-Ala^{35}]hGLP-1(7-36)NH_2
6	1.39	17.6	[Aib^{8,35},Phe^{31}]hGLP-1(7-36)NH_2
7	1.77	7.40	[Aib^8,Phe^{31},β-Ala^{35}]hGLP-1(7-36)NH_2
8	2.12	8.91	[Aib^{8,35},Arg^{26,34},Phe^{31}]hGLP-1(7-36)NH_2

[a] *The assays were done in CHO-K1 cells expressing the human recombinant GLP-1 receptor.*

novel hGLP-1 analogs with modifications at positions 8 and 35 also retain receptor potency of the native hGLP-1 (Table 1). Replacement of Trp31 by chemically more stable Phe does not significantly influence receptor affinity (compounds **6** and **7**).

The *in vivo* studies of this new series of hGLP-1 analogs in normal Sprague–Dawley rats demonstrated that the efficacy of the analogs, in terms of the glucose-dependent stimulation of insulin secretion, is highly correlated with their *in vitro* plasma half-life [3]. Among these analogs, compound **4** enhanced the insulin response to elevated glucose with a calculated ED_{50} at 16.0 pmol/kg, compared to that of the native hGLP-1(7-36)NH2 at 121 pmol/kg [4]. This 7.6-fold increase in efficacy is likely due to its enhanced enzymatic stability, resulting in an increased circulating half-life. In studies utilizing the *db/db* mouse, intraperitoneal administration of compound **4** at 5–50 nmol/kg to 5-week old animals produced a dose-dependent reduction in blood glucose monitored over a 5-h period [4].

In conclusion, we have designed and synthesized a novel class of GLP-1 analogs that have substantially enhanced plasma half-life, while retaining full receptor potency of the native hormone. The representative analog, compound **4**, is significantly more efficacious than hGLP-1 *in vivo*, and is effective in lowering blood glucose in the *db/db* mouse model of type 2 diabetes.

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TABLE 1-continued

Example Number	Mol. Wt. Expected	Mol. Wt. MS(ES)	Purity (HPLC)
376	3327.7	3327.4	98%
377	3398.8	3398.7	97.50%
378	3311.6	3311	93%
379	3366.85	3366.5	97%
380	3309.8	3309.4	99%
381	3354.8	3354.5	97.70%
382	3350.9	3350.3	97.20%
383	3311.73	3310.7	92%
384	3481.95	3481.3	94.30%
385	3281.76	3281.6	98%
386	3509.02	3509.1	99.40%
387	3665.2	3665.1	99%
388	3365.91	3365	97%
389	3324.79	3324.2	95%
390	3539	3539.2	93%
391	3381.74	3381.3	97%
392	3410.89	3409.8	99%
393	3481.95	3481.1	90%
394	3286.76	3286.2	99.20%
395	3300.76	3299.4	93%
396	3350.81	3349.4	99%
397	3400.87	3400.1	99%
398	3406.84	3406.4	99%
399	3356.77	3356.6	99%
400	3384.87	3384.43	94%

TABLE 1-continued

Example Number	Mol. Wt. Expected	Mol. Wt. MS(ES)	Purity (HPLC)
401	3400.87	3401.3	99%
402	3466.03	3466.9	97.40%
403	3522.05	3522.06	93%
404	3550.11	3550.2	98%
405	3567.09		99%
406	3763.38	3763.2	95%
407	3636.15	3635.8	99%
408	3664.21	3663.3	99%
409	3720.32	3719.5	99%
410	3692.27	3691.7	99%
411	3555.13	3554.4	99%

What is claimed is:

1. A method for treating a disease selected from the group consisting of Type I diabetes and Type II diabetes in a subject in need thereof, said method comprising administering to said subject an effective amount of a compound according to the formula [$Aib^{8,35}$]hGLP-1(7-36)NH_2(SEQ ID NO:2), or a pharmaceutically acceptable salt thereof.

* * * * *